REMARKS

The October 3, 2008 Official Action has been carefully considered. In view of the amendment submitted herewith and these remarks, favorable reconsideration and allowance of this application are respectfully requested.

At the outset, it is noted that a shortened statutory response period of three (3) months was set in the October 3, 2008 Official Action. The initial due date for response, therefore, was January 3, 2009. A petition for a one (1) month extension of the response period is included with this amendment and request for reconsideration, which is being filed before the expiration of the one (1) month extension period.

As another preliminary matter, it is noted that the restriction requirement set forth in the preceding Official Action has been maintained and made final. Applicants again respectfully submit that this requirement is improper. This is certainly true with respect to the subject matter of claims 33-35, which should be rejoined for examination in this application. There is a technical feature that is common to all of claims 22-29, 32, 36, 38, 39, 41 and 42, as well as claims 33-35. The common technical feature in the claimed screening method is the detection of the occurrence of inhibition of phosphorylation of the tau protein by the candidate CK1 inhibitor substance by identifying the specific site(s) at which CK1 phosphorylates tau protein, as recited in claim 22. This common technical feature clearly defines the contribution which the claimed invention, considered as a whole, makes over the prior art. In any case, applicants wish to make clear that their election of the Group II claims, i.e., claims 22-32, 36-41 and 51 in response to the aforementioned restriction requirement is without prejudice to their right to file one or more divisional applications, as provided in 35 USC §121, directed to any subject matter held finally withdrawn from consideration in this application.

The October 3, 2008 Official Action includes certain objections relating to formal matters. Specifically, the title of the invention is considered non-descriptive and a new more descriptive title is required. Also, objection has been made to the form of claims 27 and 28. Each of these objections has been overcome by the present amendment.

Turning to the substantive aspects of the October 3 Official Action, claims 25 and 29 stand rejected for allegedly failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. This rejection is based on the claim terminology "the

nucleic acid molecule" and "stringent conditions", which appears in claims 25 and 29.

Claims 22-29, 32, 36, 38, 39, 40 and 42 have been rejected for allegedly failing to comply with the written description and enablement requirements of 35 USC §112, first paragraph. According to the examiner, the rejected claims include derivatives of the tau protein and casein kinase 1 (CK1) which are inadequately described in the specification. Similarly, the examiner contends, in support of the insufficient enablement rejection, that the specification does not reasonably enable screening methods using any and all variants of CK1 and tau protein as broadly encompassed by the claims.

Claims 22, 23, 26, 27, 32, 36 and 38 have been rejected under 35 USC §103(a) as allegedly unpatentable over the combined disclosures of no fewer than six (6) references, namely, Meijer et al., "Inhibition of cyclin-dependent kinases, GSK-3β and CK1 by hymenialdisine, a marine sponge constituent" (Chemistry and Biology, 7: 51-63 (2000)), Chijiwa et al., "A Newly Synthesized Selective Casein Kinase I Inhibitor, N-(2-Aminoethyl)-5-chloroisoquinoline-8-sulfonamide, and Affinity Purification of Casein Kinase I from Bovine Testis*" (J. Biol. Chem., 264: 4924-4927 (1989)), Lau et al., "Tau Protein Phosphorylation as a Tehrapeutic Target in Alzheimer's Disease" (Current Topics in Medicinal Chemistry, 2: 395-415 (2002)), Castro et al.,. "Inhibition of tau phosphorylation: a new therapeutic strategy for the treatment of Alzheimer's disease and other neurodegenerative disorders" (Expert Opinion on Therapeutic Patents, 10: 1519-1527 (2000)), Singh et al., "Phosphorylation of τ Protein by Casein Kinase-1 Converts It to an Abnormal Alzheimer-Like State" (J. Neurochemistry, 64: 1420-1423 (1995)), and Kuret et al., "Casein Kinase 1 Is Tightly Associated with Paired-Helical Filaments Isolated from Alzheimer's Disease Brain" (J. Neurochemistry, 69: 2506-2515 (1997)) (hereinafter "the main obviousness rejection"). Four (4) of these references are listed in the Information Disclosure Statement which is of record in this application. In support of this rejection, the examiner has interpreted the language of claim 22 such that it calls for detecting whether or not tau protein is phosphorylated by CK1 "without requiring identification of the specific residue(s) of the tau protein that is/are phosphorylated", as stated at page 16 of the October 3 Official Action.

After summarizing the disclosures of the cited references, the examiner states, at page 18 of the October 3 Official Action that: "[t]he combination suggests screening for CK1 inhibitors of tau protein, however, there appears to be no express teaching of a method of using tau as a substrate in the screening method for CK1 inhibitors of tau phosphorylation" [emphasis in

original]. The examiner ultimately asserts that "it would have been obvious to one of ordinary skill in the art to combine the teachings of Meijer, Chijiwa, Lau, Castro, Singh and Kuret to use tau as the substrate in the method of Chijiwa [screening of CK1-7 for inhibition of CK1 using casein substrate] or Meijer [natural product hymenialdisine found to inhibit CK1 phosphorylation of presenilin-2]".

Claims 24 and 25 also stand rejected under 35 USC §103(a) as allegedly unpatentable over the same six (6) references supporting the main obviousness rejection, and further in view of Graves et al., J. Biol. Chem., 268: 6394-6401 (1993). Graves is cited for its disclosure of a nucleic acid encoding a rat testis CK1 polypeptide that has a nucleotide sequence that encodes a polypeptide that is 100% identical to SEQ ID NO. 1 in the present application, the recombinant production of such polypeptide and, according to the examiner, its use in an inhibition assay. Based on this disclosure, the examiner contends that it would have been obvious to one of ordinary skill in the art to combine the teachings of Meijer, Chijiwa, Lau, Castro, Singh, Kuret and Graves and to use the polypeptide of Graves in a CK1 inhibitor screening method with a tau protein as substrate.

Claims 28 and 29 have also been rejected under 35 USC §103(a) as allegedly unpatentable over the same six (6) references supporting the main obviousness rejection, and further in view of U.S. Patent 6,593,512 to Vitek et al. Vitek is relied on for its disclosure of cloning a nucleic acid encoding a human tau polypeptide that has a nucleotide sequence that encodes a polypeptide that is 100% identical to SEQ ID NO.1 in the present application, as well as the recombinant production of such polypeptide and, according to the examiner, its use in an inhibition assay. Based on this disclosure, the examiner asserts that it would have been obvious to one of ordinary skill in the art to combine the teachings of Meijer, Chijiwa, Lau, Castro, Singh, Kuret and Vitek and to use the polypeptide of Vitek in a CK1 inhibitor screening method with tau protein as substrate.

Claims 39, 41 and 42 have been further rejected under 35 USC §103(a) as allegedly unpatentable over the same six (6) references supporting the main obviousness rejection and further in view of Zhu et al., Current Opinion in Chemical Biology, 5: 40-45 (2001). Zhu purports to disclose the use of protein chips for protein kinase assay by, for example, attaching a substrate to a microwell plate and assaying kinase activity, and to suggest that in conjunction with mass spectrometric identification, protein chips might have wide application in drug

discovery. Based on this combination of references, the examiner urges that it would have been obvious to one of ordinary skill in the art to combine the teachings of Meijer, Chijiwa, Lau, Castro, Singh, Kuret and Zhu and to use a protein chip and mass spectroscopy in a CK1 inhibitor screening method with tau protein as substrate.

The October 3, 2008 Official Action also includes, at page 23-27, a series of obviousness-type double patenting rejections which essentially track the aforementioned 35 USC §103 rejections. The rejections rely primarily on claims 6-9 and 12 of U.S. Patent 5,994,084 to Anderton et al. (hereinafter "the '084 patent"), with additional reliance on the same combinations of references cited in support of the several §103 rejections noted above.

The foregoing objections and rejections constitute all of the grounds set forth in the October 3, 2008 Official Action for refusing the present application.

Applicants, through their undersigned attorneys, requested a telephone interview with Examiner Steadman, which was held January 22, 2009. The courtesy extended to applicants' attorneys in granting the interview is appreciated. The purpose of the interview was to discuss a proposed amendment of claim 22 with Examiner Steadman, and in particular to point out the reasons why the amended version of claim 22 is believed to comply with the recently promulgated PTO Written Description Training Materials.

It was also mentioned, with respect to all of the prior art rejections and obviousness-type double patenting rejections, that many of the tau protein phosphorylation sites recited in the pending claims were unknown prior to the present invention.

It was further stated that the enablement rejection would be argued, as it is considered improper with respect to the claims as originally presented.

The possibility of rejoining claims 33-35 for examination in this application was also brought up for discussion.

At the conclusion of the interview, Examiner Steadman indicated that further consideration would be required to determine whether the proposed claim amendment would overcome the written description, scope of enablement, obviousness and obviousness-type double patenting rejections in the Official Action outstanding.

In accordance with the present amendment, the specification has been amended to substitute a new title of the invention, in accordance with the examiner's suggestion.

As for the claim amendments, claim 22 has been amended to include the subject matter of

original claims 24 and 28. Specifically, CK1 is further characterized as having greater than 80% sequence identity with full length CK1 that has the amino acid sequence set out between amino acids 1 and 428 inclusive of SEQ ID NO.1; and the tau protein is further characterized as having greater than 80% sequence identity with full length tau protein that has the amino acid sequence set out between amino acids 1 and 441 inclusive of SEQ ID NO.2.

Support for this amendment is provided in the paragraph bridging pages 17-18 of the present specification. Claims 24 and 28 have been cancelled as a consequence of the amendment of claim 22.

Furthermore, claim 22, as now amended, positively calls for "identifying the site(s) at which the casein 1 phosphorylates the tau protein . . . and S435 of tau protein". Support for this added recitation is provided in the paragraph bridging pages 25-26 of the present specification.

Also included with the present amendment is new claim 55, which is drawn to a preferred group of tau protein phosphorylation sites. Support for this claim is provided in Table 2 at pages 43-44 of the specification, in which the group members are identifiable as sites at which phosphorylation of the tau protein actually occurs (see col. 1), new CK1 phosphorylation sites (see col. 2) and sites not previously known to be phosphorylated by another kinase (see all other columns with "kinase" headings).

The present amendment also cancels claims 25 and 29. The cancellation of these claims is without acquiescence in the propriety of the rejections thereof set forth in the October 3 Official Action and without prejudice to applicants' right to file a continuing application directed to the subject matter of the cancelled claims, as provided in 35 USC §120.

No new matter has been introduced into this application by reason of the present amendment, entry of which is hereby respectfully requested.

As a result of this amendment, the formal objections stated in the October 3 Official Action have been overcome, and the 35 USC §112, second paragraph, rejection of claims 25 and 29, the 35 USC §112, first paragraph, of claims 22-29, 32, 36, 38, 39, 40 and 42 for alleged inadequate written description and the main obviousness rejection and related obviousness-type double patenting rejection of claims 22, 23, 26, 27, 32, 36 and 38 cannot be maintained. In this connection, it is noted that the cancellation of claims 25 and 29 renders moot the rejection of those claims for alleged indefiniteness. Also, the main obviousness rejection, as well as the separate obviousness rejection based on the combined disclosures of Meijer, Chijiwa, Lau,

Castro, Singh and Kuret and Zhu et al., were not applied in rejecting claims 24 and 28, the subject matter of which is now incorporated into claim 22. Furthermore, although Zhu contemplates coupling protein arrays with mass-spectrometric analysis in drug discovery, the reference provides no teaching or suggestion of the use of mass spectrometry to measure specific tau phosphorylation sites to determine whether an inhibitor has prevented phosphorylation. Indeed, Zhu is completely silent on the use of mass spectrometry for this purpose, and it would have been far from obvious that such methods could be used in the context of the screening method claimed by applicants.

Moreover, the amendment of claim 22 with regard to sequence identity with full length CK1 and with full length tau protein is in complete conformity with the PTO's Written Description Training Materials, Revision 1, March 25, 2008. See also, *Ex parte Kubin*, 83 USPQ2d 1410 (PTO BPAI 2007).

Thus, the only matters remaining to be addressed are the 35 USC §112, first paragraph, rejection of claims 22-29, 32, 36, 38, 39, 40 and 42 for alleged insufficient enablement, the obviousness rejection of claims 24 and 25 based on the combined disclosures of Meijer, Chijiwa, Lau, Castro, Singh, Kuret and Graves, the related obviousness-type double patenting rejection of claims 24 and 25, the obviousness rejection of claims 28 and 29 based on the combined disclosures of Meijer, Chijiwa, Lau, Castro, Singh, Kuret and Vitek, and the related obviousness-type double patenting rejection of claims 28 and 29. For the reasons given below, these last-mentioned grounds of rejection are respectfully traversed.

A. The Impropriety of the 35 USC §112, First Paragraph Rejection of Claims 22-29, 32, 36, 38, 39, 40 and 42 Based on Alleged Insufficient Enablement

Applicants respectfully take exception to the examiner's contention that the specification does not enable the skilled artisan to make the invention commensurate in scope with original claims 24 and 28. As noted above, the subject matter of claims 24 and 28 has been added by amendment to claim 22. Applicants' specification is more than adequate to enable the skilled artisan to practice the full scope of the rejected claims.

It is important to recognize that the claims currently under consideration are not drawn to a nucleic acid, as such. Rather, the claims call for a screening method utilizing CK1 that is

capable of phosphorylating phosphorylation sites of tau protein, and tau protein which functions as a substrate for such phosphorylation. Notably, the examiner acknowledges at page 10 of the October 3 Official Action that the present specification is "enabling for methods using art-recognized CK1 and tau proteins". These would include, at least, human CK1α, β, γ, δ forms, both natural and recombinantly expressed (see Graves), bovine brain and kidney CK1 and yeast CK1 (see Singh) as the CK1 variants, and bovine (see Singh), murine (see the '084 patent to Anderton and Miller) and human tau (see Lau) as tau protein variants.

The examiner evidently has reservations about the sufficiency of enablement insofar as any and all variants of CK1 and tau protein are concerned. However, neither original claims 24 and 28, nor the presently amended claims include any and all such variants. The claims expressly require that any CK1 variant have greater than 80% sequence identity with the full length CK1 having the amino acid sequence set out between amino acids 1 and 428 inclusive of SEQ ID NO:1, and that any tau protein variant have greater than 80% sequence identity with the full length tau protein having the amino acid sequence set out between amino acids 1 and 441 inclusive of SEQ ID NO:2. Thus, the issue boils down to whether, given applicants' specification, any person skilled in the art would be able to practice applicants' screening method as broadly as it is now claimed without undue experimentation. Taking into account the various factors set out in *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988), this issue must be resolved in applicants' favor.

The breadth of the recitations relating to CK1 and tau protein in the claimed screening method has been stated above. Those skilled in the art would have no difficulty identifying CK1 or tau protein variants within the scope of amended claim 22, or employing any of them in the screening method claimed by applicants. Considering the nature of the invention, the rejected claims cannot reasonably be regarded as a unduly broad, particularly given the familiarity of those skilled in the art with methods of screening for inhibitors of CK1 activity that were known in the art at the time of the present invention.

In *Wands*, the court determined that the level of skill in the field of molecular biology is high. *Id.* at 1406. Moreover, those skilled in the art would be expected to be familiar with previously disclosed methods of screening for CK1 inhibitors, such as Mashhoon et al., J. Biol. Chem. (2000) (copy attached).

Predictability is of little or no consequence when, as in this case, routine testing reveals

whether or not a given CK1 or tau protein variant satisfies the requirements of the claimed screening method.

As for the amount of direction provided by the inventors, the present specification provides detailed disclosure regarding the suitability of CK1 and tau protein variants for use in the claimed invention. This includes, *inter alia*, numerous serine or threanine residues of tau protein that serve as phosphorylation sites, many of which are described in the present specification for the first time, as well as means for identifying the occurrence of phosphorylation at such sites.

Regarding working examples, see pages 34-37 of the specification.

The quantity of experimentation needed to determine the feasibility of using the full scope of CK1 and tau protein variants, as presently claimed in applicants' screening method, may be regarded as extensive. However, the nature of the experimentation is quite routine and well within the capabilities of those skilled in the art. The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. *In re Angstadt*, 190 USPQ 214, 219 (CCPA 1976). To the same effect is *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (ITC 1983), *aff'd. sub. nom, Massachusetts Institute of Technology v. A.B. Fortia*, 227 USPQ 428 (Fed. Cir. 1985), in which it was observed that the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation.

The foregoing review of the "Wands" factors plainly establishes that the present specification fully satisfies the enablement requirement of 35 USC §112, first paragraph, with respect to the screening method of claims 22-29, 32, 36, 38, 39, 40 and 42.

For all of the above reasons, the rejection of claims 22-29, 32, 36, 38, 39, 40 and 42 for allegedly failing to satisfy the enablement requirement of 35 USC §112, first paragraph, is improper and should be withdrawn upon reconsideration.

B. The Impropriety of the 35 USC §103(a) Rejections of Claims 22-29, 32, 36 and 38 as Allegedly Obvious Over Prior Art

It is well-settled that the examiner bears the initial burden of establishing a *prima facie* case of obviousness under 35 USC §103. *Ex parte Wolters*, 214 USPQ 735 (Bd. Apps. 1979). Furthermore, an obviousness determination under 35 USC §103 based on a combination of prior

art references is proper only when there is an apparent reason to combine known elements of the prior art in the fashion set forth in the claims at issue. *KSR Intl. Co. v. Teleflex, Inc.*, 82 USPQ2d 1385 (2007). In the present case, the rejection of claims 22-27, 32, 36 and 38 based on the combined disclosures of Meijer, Chijiwa, Lau, Castro, Singh, Kuret and Graves is plainly improper because it is devoid of any rational basis to support the legal conclusion that the screening method recited therein would have been *prima facie* obvious. The same is true with respect to the §103(a) rejection of claims 22, 23, 26-29, 32, 36 and 38 based on the combined disclosures of Meijer, Chijiwa, Lau, Castro, Singh, Kuret and Vitek.

The rationale given by the examiner for combining these particular groups of references in support of these rejections is dubious at best. The cited references make clear that the exact role of any candidate tau kinases in Alzheimer's disease "is not yet understood" (Castro et al. at 1520, col. 1, lines 40-43) and Lau et al. conclude "the conviction of any one of these suspect kinases as guilty parties in mediating tau pathology will depend upon the development of highly specific inhibitors that are efficacious in reducing tau pathology in animal models and, ultimately, in AD patients" (at 399, col. 1, lines 12-16). Such statements in the references cited in support of the present rejections clearly render the examiner's certainty in asserting that CK1 was a valid therapeutic target rather presumptuous.

1. The Combination of Meijer, Chijiwa, Lau, Castro, Singh and Kuret Considered in View of Graves Fails to Render the Subject Matter of Claims 24 and 25 *Prima Facie* Obvious

There is no apparent reason for combining Graves with the other six (6) references in the manner proposed by the examiner. Applicants do not dispute that Graves discloses a cDNA encoding a 428 amino acid polypeptide, designated CK1δ, which is identical to that presently recited in claim 22. However, one searches Graves in vain for any disclosure or suggestion of a practical utility for the CK1δ described therein. It is disingenuous to assert that Graves teaches the use of CK1δ in an inhibition assay when, in fact, Graves only discloses an assay in which a known inhibitor of CK1 was used to characterize the cDNA, and not to screen for candidate CK1 inhibitors.

2. The Combination of Meijer, Chijiwa, Lau, Castro, Singh and Kuret Considered in View of Vitek Fails to Render the Subject Matter of Claims 28 and 29 *Prima Facie* Obvious

Vitek provides no teaching or suggestion of using human tau protein produced by the transgenic mouse disclosed therein in a screening assay for inhibitors of any kinase, much less CK1 inhibitors. There is nothing in Vitek's disclosure to support the examiner's contrary assertion at page 21 of the October 3 Official Action[1]. Vitek is concerned solely with a transgenic mouse, which expresses the human tau gene, and which is disclosed as being useful as a source of human tau protein and as a model of Alzheimer's disease, Frontal Tempral Dementia and Parkinson's-like diseases.

3. The Cited References Fail to Teach or Suggest All of the Recitals of the Rejected Claims

Even it were assumed that the cited references can reasonably be combined as contended by the examiner (which the applicants vigorously dispute), the resulting combination nevertheless fails to satisfy all of the recitals of the rejected claims.

As noted above, the amended claims now positively call for identifying the site(s) at which the CK1 phosphorylates the tau protein, followed by a Markush group of such sites. Not one of the references applied in rejecting claims 22-29, 32, 36 and 38 appears to teach or suggest this feature of applicants' screening method. Applicants set out to find a solution to the problem of providing better screening tools able to more accurately predict therapeutically efficacious inhibitors of CK1. The problem was solved, in accordance with the present invention, through the provision of a number of previously undescribed phosphorylation sites in tau protein that are found in pathological forms of PHF tau, but which are not found to be phosphorylated in normal tau, and means for quantifying the level of phosphorylation at each site. The sites set forth in the amended claims were not described or suggested in the prior art. See, in particular, claims 32 and 55.

The conclusion is inescapable, therefore, that applicants' improved method of identifying specific inhibitors of CK1-mediated phosphorylation at specific sites on tau protein is patentably

1 The specific citation concerning the use of Vitek's tau protein in an inhibition assay, at page 21 of the October 3 Official Action, appears to be a citation to Graves, i.e., page 6398, Fig. 6.

distinguishable over the references of record.

In view of the examiner's failure to provide any reasonable basis for concluding that the combined disclosures of Meijer, Chijiwa, Lau, Castro, Singh, Kuret and Graves render claims 22-27, 32, 36 and 38 *prima facie* obvious, the 35 USC §103(a) rejection of those claims based on such combination of references should be withdrawn upon reconsideration. Similar reasoning compels the conclusion that the §103(a) rejection of claims 22, 23, 26-29, 32, 36 and 38 over the combined disclosures of , Chijiwa, Lau, Castro, Singh, Kuret and Vitek is likewise improper and should be withdrawn upon reconsideration. Citing references which merely indicate that isolated elements in the claims are known is not a sufficient basis for concluding that the combination of claimed elements would have been *prima facie* obvious. *Ex parte Hiyamizu,* 10 USPQ 1393, 1394 (PTO BPAI 1988). To the same effect is *Ex parte Levengood,* 28 USPQ 1300 (PTO BPAI 1993) (examiner cannot establish obviousness by locating references which describe various aspects of applicant's invention without also providing evidence of the motivating force which would impel one skilled in the art to do what applicant has done). Here, as in *Levengood,* the references cited as evidence of obviousness "fall short of providing the 'motivation' or 'suggestion' to assemble their teachings into a viable process". *Id.* at 1302.

These grounds of rejection should be reversed for the additional reason that the prior art references cited as evidence of obviousness fail to satisfy all of the recitals of the rejected claims.

C. The Impropriety of the Several Obviousness-Type Double Patenting Rejections

Obviousness-type double patenting is a judge-made doctrine based on public policy, which has as its objective the prevention of unjustified or improper time-wise extension of the right to exclude conferred by a U.S. patent. This policy is effectuated by refusing issuance of separate patents on applications that claim obvious variations of the same invention. In this case, however, there is no possibility for unjustified or improper time-wise extension of applicants' patent rights because the present claims cannot possibly constitute an obviousness variation of the method for testing therapeutic agents for treating Alzheimer's disease claimed in claims 6-9 and 12 of the '084 patent.

The testing method claimed in claims 6-9 and 12 of the '084 patent is a cell-based method involving the use of a transgenic cell comprising, *inter alia*, a DNA sequence encoding a kinase

selected from the group consisting of glycogen synthase kinase-3α (GSK-3α) and glycogen synthase kinase-3β (GSK-3β). See the reference to "a cell as claimed in claim 1", in the first step of the method of claim 6. By contrast, the claims of the present application are drawn to a method of screening substances capable of inhibiting CK1 phosphorylation of tau protein which requires contacting candidate substance, tau protein and CK1 and identifying the occurrence of phosphorylation at the specific sites recited in claim 22. In view of these clear differences, the grant of a patent on the present application could not conceivably result in a time-wise extension of the '084 patent grant.

Furthermore, the various obviousness-type double patenting rejections set forth in the October 3 Official Action are unfounded for at least the same reasons given above in addressing the impropriety of the §103 rejections of claims 22-27, 32, 36 and 38 based on Meijer, Chijiwa, Lau, Castro, Singh and Kuret, considered in view of Graves, and of claims 22, 23, 26-29, 32, 36 and 38 based on Meijer, Chijiwa, Lau, Castro, Singh and Kuret considered in view of Vitek. Clearly, the '084 patent fails to compensate for the fundamental deficiencies in the combinations of references cited as evidence of obviousness in support of these rejections.

For all of the reasons stated above, the obviousness-type double patenting rejections set forth in the October 3, 2008 Official Action are improper and should, therefore, be withdrawn upon reconsideration.

Lastly, the examiner is correct in presuming (at the top of page 15 of the October 3 Official Action) that the subject matter of the various claims was commonly owned at the time the inventions covered thereby were made.

In view of the present amendment and the foregoing remarks, it is respectfully urged that the objections and rejections set forth in the October 3, 2008 Official Action be withdrawn and that this application be passed to issue, and such action is earnestly solicited.

Respectfully submitted,

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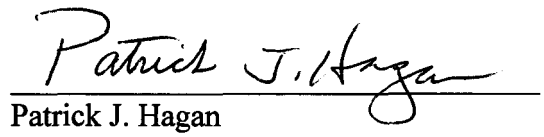

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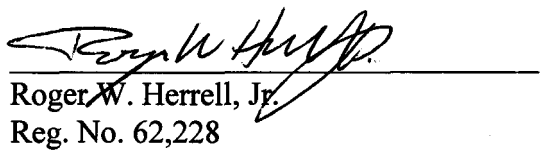

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Attachments:
1. Mashhoon et al. (2000)

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Crystal Structure of a Conformation-Selective Casein Kinase-1 Inhibitor*

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Summary

Members of the casein kinase-1 family of protein kinases play an essential role in cell regulation and disease pathogenesis. Unlike most protein kinases, they appear to function as constitutively active enzymes. As a result, selective pharmacological inhibitors can play an important role in dissection of casein kinase-1 dependent processes. To address this need, new small-molecule inhibitors of casein kinase-1 acting through ATP-competitive and ATP-noncompetitive mechanisms were isolated on the basis of *in vitro* screening. Here we report the crystal structure of 3-[(2,4,6-trimethoxyphenyl)methylidenyl]-indolin-2-one (IC261), an ATP-competitive inhibitor with differential activity among casein kinase-1 isoforms, in complex with the catalytic domain of fission yeast casein kinase-1 refined to a crystallographic R-factor of 22.4% at 2.8 Å resolution. The structure reveals that IC261 stabilizes casein kinase-1 in a conformation midway between nucleotide substrate liganded and nonliganded conformations. We propose that adoption of this conformation by casein kinase-1 family members stabilizes a delocalized network of side-chain interactions and results in a decreased dissociation rate of inhibitor.

Introduction

Mammalian casein kinase-1 (CK1) [1] is a protein kinase family consisting of multiple isoforms encoded by distinct genes (Ckiα, β, γ1, γ2, γ3, δ, ε). Family members contain a highly conserved ~290 residue N-terminal catalytic domain coupled to a variable C-terminal region that ranges in size from 40 to 180 amino acids (1). The C-terminal region serves to promote differential subcellular localization of individual isoforms (2, 3) and to modulate enzyme activity (4).

Though their biological function is not understood in molecular detail, recent evidence suggests CK1 isoforms play a role in regulation of DNA-repair (5, 6), cellular morphology (7), circadium rhythm (8), and stabilization of cellular proteins such as β-catenin (9, 10) and membrane-bound transporters (11) against degradation. These phenotypes, combined with observations that different CK1 homologs are essential for intracellular trafficking in different cellular compartments (12 - 14), suggest that CK1 isozymes directly influence protein turnover and other transport-dependent cellular processes such as autophagy and secretion.

In addition to their roles in normal cell biology, at least one CK1 isoform, Ckid, has been implicated in the pathogenesis of Alzheimer's disease (15, 16). Its colocalization with tau-containing neuronal inclusions (neurofibrillary tangles and Pick bodies), but not tau negative inclusions (Lewy bodies, Hirano bodies, and Marinesco bodies), is consistent with this isoform participating in the pathological hyperphosphorylation of tau protein in a range of neurodegenerative diseases (17). Indeed, the highly elevated levels of Ckid found in AD, and its association with hallmark lesions of neurodegeneration, suggest it is part of a final pathway of degeneration common to AD, progressive supranuclear palsy, and amyotrophic lateral sclerosis (17).

In light of these findings, selective and potent inhibitors of CK1 are needed to help resolve the role of CK1 homologs in cell regulation, and may have utility in the treatment of neurodegenerative disease. Two selective CK1 inhibitors have been reported. The first is N-(2-aminoethyl)-5-chloroisoquinoline-8-

sulfonamide, also known as CKI7 (18). It is one of a large family of naphthalene and isoquinoline sulfonamide derivatives that compete with nucleotide substrate binding, and that differ in potency and selectivity among protein kinases (reviewed in Ref. 19). As a result, individual isoquinoline sulfonamides have emerged as popular pharmacological tools for inhibiting CK1 activity in broken cell preparations. Nonetheless, the presence of a primary amine moiety on CKI7 renders it charged at physiological pH and of poor utility in intact cells and tissues. Other selective CK1 inhibitors are ribofuranosyl-benzimidazoles (20). Some of these are potent inhibitors of both CK1 and casein kinase-2, but also inhibit RNA polymerase. Relative selectivity among CK1 isoforms has not been shown with either CKI7 or the ribofuranosyl-benzimidazoles.

Using a C-terminal truncation mutant (Cki1Δ298) of Cki1 (one of four CK1 isoforms in fission yeast), we recently determined the structural basis of CK1 substrate selectivity and regulation using X-ray crystallography (21, 22). Subsequently the structure of Cki1Δ298 in complex with CKI7 was solved to clarify how the inhibitory selectivity of CKI7 and other isoquinoline sulfonamide inhibitors was achieved (23). Here we report the use of high-throughput screening of a library of small molecules to obtain a novel CK1-selective inhibitor, IC261, and its structure in complex with Cki1Δ298 determined by X-ray crystallography. The results show that IC261 is an ATP-competitive inhibitor that stabilizes Cki1Δ298 in an inactive conformation. Because of its affinity and neutral charge, IC261 can serve as a new generation of CK1 inhibitor potentially active in intact cells.

Experimental Procedures

Materials. CK1 isoforms from *S. pombe* (Cki1Δ298) and human (Ckiα$_1$, Ckiδ, and Ckiε) were prepared as described previously (15, 16, 21). Bovine heart PKA was prepared as previously described (24) and kindly supplied by Dr. John Scott (Oregon Health Sciences University), *P. ochraceus* cyclin-dependent protein kinase p34^{cdc2}, bovine thymus p55^{fyn}, and their substrate peptides were purchased from Upstate Biotechnology, Inc. Aluminum-backed silica gel (0.25-mm) plates were purchased from EM Separation Technologies. Deuterated solvents were purchased from Aldrich. A small molecule library suitable for random screening was assembled from commercial sources and in-house chemistry resources at ICOS Corp. The library included IC261. Coordinates for models of Cki1Δ298 in complex with MgATP (1CSN) and CKI7 (2CSN), for truncated mammalian Ckiδ apoenzyme (1CKJ), and for FGFR kinase in complex with inhibitors SU5402 (1FGI) and SU4984 (1AGW) were obtained from the protein databank, whereas FGFR kinase in complex with AMP-PCP (25) was provided by Dr. S.R. Hubbard (New York University, New York, NY).

Phosphotransferase Assays. Casein kinase activity was assayed at 37°C as described previously (2). The standard reaction (40 µl) contained 25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, 50 mM NaCl, 15 mM $MgCl_2$, 2 mg/ml casein, 2 mM EGTA, 100 µM [γ-^{32}P]ATP (100 - 400 cpm/pmol). Initial velocity measurements were carried out in duplicate with ATP as the varied substrate. Kinetic constants and their standard errors were calculated as in (26). For assay of inhibitor potency (IC_{50}), [γ-^{32}P]ATP was held constant (10 µM) while IC261 concentration was varied (0.1, 0.3, 1, 3, and 10 µM). To assess kinetic mechanism, inhibitors were held constant (IC261, 20 µM; IC3608, 100 µM) while [γ-^{32}P]ATP was varied as above. For screening small molecule libraries, CK1 isoforms (Ckiα$_1$, δ, and ε) were assayed as above except that casein was used at 10 mg/ml, [γ-^{32}P]ATP was held constant at 2 µM or 1 mM.

PKA, p34^{cdc2}, and p55^{fyn}, were assayed using the phosphocellulose method (27) and synthetic

peptide substrates Kemptide (33 μM in 5 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT; Ref. 28), PKTPKKAKKL (20 μM in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT; Ref. 29), and KVEKIGEGTYGVVYK (20 μM in 20 mM HEPES, pH 7.2, 10 mM $MnCl_2$, 1 mM DTT; Ref. 30), respectively. Assays contained 10 μM [γ-^{32}P]ATP and variable concentrations of inhibitors. The resultant data was fit to the relationship:

$$A = A_0 + (A_{max} - A_0)/[1 + 10^{(\log IC_{50} - \log X}]$$

where A and A_0 are blank-adjusted protein kinase activity in the presence and absence of inhibitor (at concentration X), respectively. IC_{50} values were calculated using the nonlinear regression algorithm of GRAPHPAD PRISM (GraphPad Software Inc.).

Crystallization and Data Collection. Purified Cki1Δ298 was concentrated to 12 mg/ml and exchanged into Buffer A (10 mM MOPS, pH 7.0, 0.1 mM EGTA, 0.1% 2-mercaptoethanol and 100 mM NaCl) using centrifugal filtration. Cki1Δ298:IC261 binary complex was prepared by adding 25 mM inhibitor dissolved in DMSO to purified protein to yield a final inhibitor concentration of 500 μM, a final DMSO concentration of 2% (v/v), and an inhibitor:protein molar ratio ≈1.7:1. The addition was made in small aliquots over 30 min at room temperature to overcome the poor aqueous solubility of IC261 and to ensure binary complex formation. Binary complexes were then subjected to crystallization conditions at 16°C by vapor diffusion as described previously (21), except that final reservoir solution contained 1.5 – 1.6 M $(NH_4)_2SO_4$, 5 mM sodium acetate, pH 4.2, and 1% 2-methyl-2,4-pentanediol. These conditions yielded two crystal morphologies: Hexagonal rods (0.28 x 0.1 x 0.1 mm, space group $P6_1$ with cell dimensions $a = b = 113.5$ Å, $c = 110.4$ Å) that diffracted to 2.8 Å, and hexagonal bipyramids (cell dimensions $a = b = 96.4$ Å, $c = 214.4$ Å) that diffracted to 4.0 Å when final DMSO concentration was kept <1%. Although these latter crystals grew large, they were consistently mosaic and were not analyzed further.

Binary complex crystals were harvested using a nylon loop, brushed against cryoprotection solution (5 mM sodium acetate, pH 4.2, 2 M $(NH_4)_2SO_4$, 30% sucrose) and flash frozen in a dry nitrogen stream. All data were collected at -140°C on a Rigaku R-AXIS IIc image plate system, mounted on a Rigaku RU200-HB rotating anode X-ray generator (Cu Kα) operated at 50 kV and 100 mA. Data were processed with BIOTEX (Rigaku Intl. Corp.) and DENZO/SCALEPACK (31) program packages.

Structure Determination and Refinement. A molecular replacement solution was found using data between 15 – 3.5 Å, the program AMORE (32), and coordinate set 2CSN as the search model. Initially, data was processed in space group $P6_2$ with one molecule in the asymmetric unit and cell dimensions $a = b = 113.5$ Å and $c = 55.2$ Å. The rotation function yielded only one peak and the translation search with that orientation yielded a trial solution with correlation coefficient of 58.5% and R_{cryst} of 38.4%. When further refinement using all data to 2.8 Å failed to improve the R_{cryst}, the diffraction pattern was examined more closely, revealing additional reflections consistent with a double cell along the *c*-axis. Data were then reprocessed in space group $P6_1$ with cell dimensions $a = b = 113.5$ Å and $c = 110.4$ Å, and reanalyzed in AMORE. Again the rotation function yielded a single peak. During a two-body translation search, two molecules were found in the asymmetric unit shifted by ≈1/2 *c* (translation vector -1.43, 0.54, 53.8), and related by a 4° rotation about crystal axis *b*. The correlation coefficient for the correct solution was 66.0% with an R_{cryst} of 36.6%. All successive refinement was done using the program CNS (33), using standard restraints and setting 10% of the data aside to calculate the R_{free} value (34). Rigid body refinement (treating each molecule in the unit cell as a rigid body), was followed by 10 cycles of simulated annealing. After several cycles of refinement, Fourier maps were calculated and model quality was visually assessed within TURBO-FRODO (35, 36). Solvent molecules were then picked automatically and the correctness of the assignment was examined by visualizing electron density maps. At this point, Fourier maps (both $2F_O - F_c$ and $F_O - F_c$ maps) showed strong continuous density for the inhibitor. Initial models of IC261 (both E and Z geometric isomers) were generated using TURBO-FRODO, subjected to energy minimization with X-PLOR2D (37), then manually fit into

the density. Topology and parameter files were created for inhibitor and it was refined along with protein through subsequent cycles of refinement.

Structure Analysis. Nomenclature for amino acid residues, loops, and secondary structure elements was as in (23). The final model was characterized using the program PROCHECK (38). Atomic coordinates derived from different models were superpositioned using X-PLOR (39). Mainchain atoms (N, Cα, Cβ, O) corresponding to secondary structure elements within the N-terminal (β–strands 3-5 and α-helix A) or the C-terminal (α-helices B - I) domains were used separately to align CK1 models. Domain movements were quantified from superpositioned coordinate sets using HINGEFIND (40).

Analytical Methods. Circular dichroism measurements were performed in the presence (10, 30, and 50 μM) or absence of IC261 using a Jasco 720 CD spectropolarimeter. Cuvette pathlength and sample concentration were adjusted in diluent (5 mM sodium phosphate, pH 7.0, 50 mM $NaClO_4$) so that the total optical density was less than 1.0 over the measured spectral range (190 - 260 nm). Secondary structure content was calculated using the program SELCON (41).

Equilibrium denaturation measurements were performed on Cki1Δ298 (0.25 mg/ml) in darkness at room temperature overnight in 0.2 M NaCl, 10 mM HEPES, pH 7.4, and 0 - 7 M urea in the presence and absence of saturating concentrations of IC261 (100 μM). Intrinsic Trp/Tyr fluorescence was measured in an SLM 8000c fluorometer over the emission range 300 - 400 nm upon excitation at 280 nm. Data were analyzed as in (42).

NMR spectra of IC261 were recorded using a Bruker DPX 250 MHz NMR spectrometer. Tetramethylsilane was used as an internal standard and chemical shifts were recorded in parts per million (δ) downfield from this standard.

Geometric isomers of IC261 were resolved by thin-layer chromatography on silica-gel plates using ethyl acetate/hexane (1:1) as solvent and visualized with ultraviolet light.

Statistical errors are reported as standard deviations unless otherwise noted.

Results

IC261 is a CK1-selective Protein Kinase Inhibitor - To identify candidate CK1 antagonists, a library of small molecules was screened for inhibitory activity against three mammalian isoforms of CK1 (Ckiα$_1$; Ckiδ; Ckiε) in the presence of low (2 μM) and high (1 mM) concentrations of nucleotide substrate (ATP). Two broad classes of inhibitor were identified from this screen. Class I consisted of molecules whose inhibitory activity was attenuated at high ATP concentration, suggesting they acted through the nucleotide binding site. In contrast, Class II molecules inhibited protein kinase activity independently of ATP concentration, consistent with an alternative mechanism of inhibition.

The structure of IC261, a CK1-selective Class I molecule identified by the screen, is shown in **Fig. 1A**. It is a 3-substituted indolin-2-one derivative. Molecules of this family are commonly prepared by base-catalyzed condensation of aldehydes and oxindole (43, 44), and therefore typically consist of mixtures of E and Z geometric isomers. When subjected to thin layer chromatography, IC261 resolved into two principal species in ~2:1 ratio, suggesting that it too was a mixture of geometric isomers (**Fig. 1B**). NMR analysis revealed a complex pattern consistent with a mixture of two species in a ~2:1 ratio (data not shown). The relative signal intensity associated with the presumptive olefinic proton (7.76 ppm for E isomer) was consistent with the E isomer being the predominant geometric isomer in the preparation (data not shown).

To assess relative selectivity, the inhibitory profile of IC261 was extended to include three unrelated protein kinases that were well characterized structurally and enzymologically: PKA, p34^{cdc2}, and *src* homolog p55^{fyn} (**Fig. 2, *top***). When assayed at 10 μM ATP, IC261 was most potent against Ckiδ (IC_{50} = 1.0 ± 0.3 μM) and Ckiε (IC_{50} = 1.0 ± 0.4 μM), followed by Ckiα$_1$ (IC_{50} = 16 ± 5 μM), followed by PKA, p34^{cdc2}, and p55^{fyn} (IC_{50}s > 100 μM). Together these data suggest that one or both geometric isomers of IC261 selectively inhibit CK1 isoforms compared to unrelated protein kinases represented in

the test group. Moreover, the results obtained from this pilot screen suggest that it is possible to isolate inhibitors with an order of magnitude selectivity for individual CK1 isoforms.

IC261 is a Competitive Inhibitor of Nucleotide Substrate - As a Class I inhibitor, IC261 was predicted to inhibit protein kinases by competing with the binding of ATP substrate. To test this hypothesis, the ability of IC261 to inhibit Cki1Δ298, a truncation mutant of *S. pombe* Cki1 (21), was characterized by steady-state kinetics. This particular CK1 isoform was chosen for analysis because it consists of only the catalytic domain of CK1 and because its structure is known at high resolution in complex with ATP (22) and at medium resolution in complex with an ATP-competitive isoquinoline sulfonamide inhibitor (23). In the absence of IC261, Cki1Δ298 returned a K_m for ATP of 25.6 ± 0.2 μM, and a V_{max} of 3.7 ± 0.2 μmol/min/mg (**Fig. 2, *bottom***). In the presence of IC261, the V_{max} changed little (3.1 ± 0.2 μmol/min/mg) whereas the apparent K_{mATP} increased to 111 ± 0.2 μM. This pattern is identical to those exhibited by ATP-competitive agents such as the isoquinoline sulfonamide CKI7 (18, 23). In contrast, IC3608, a representative Class II inhibitor, lowered V_{max} (1.6 ± 0.2 mg/min/mg) but had little effect on the K_{mATP} (30.2 ± 0.3 μM). These results confirm that IC261 inhibition is competitive with respect to ATP.

To determine whether IC261 binding was accompanied by changes in CK1 secondary structure, circular dichroism measurements were performed on Cki1Δ298 in the presence and absence of nucleotide or IC261 ligand. The resultant spectra were virtually identical in all cases, suggesting that binding of IC261 to CK1 was not accompanied by measurable changes in Cki1Δ298 secondary structure (data not shown). Consistent with this observation, the sensitivity of Cki1Δ298 to urea denaturation was unchanged in the presence or absence of IC261. Half maximal denaturation was observed in ≈3.3 M urea, yielding an extrapolated free energy of folding of 11.2 ± 0.79 kcal/mol (data not shown). Together these data suggest that one or both geometric isomers of IC261 bound in the ATP binding cleft of Cki1Δ298, and that binding did not induce measurable changes in protein secondary structure.

Crystallization of IC261 in Complex with Cki1Δ298 - To clarify the mechanism of IC261 selectivity, it was crystallized in complex with Cki1Δ from solutions containing ammonium sulfate at acidic pH. Although the binary complex of Cki1Δ:MgATP forms highly ordered trigonal crystals under these conditions (21), no crystals of this space group were formed from Cki1Δ298:IC261 binary complex. Instead, hexagonal rods of space group $P6_1$ that diffracted to 2.8 Å resolution were consistently obtained. Complete native datasets were collected from individual crystals and the structure solved by molecular replacement. Data collection and refinement statistics are presented in **Table I**. The final model contained two Cki1Δ298 molecules in the asymmetric unit (termed molecules A and B) arranged head-to-tail (**Fig. 3**). As in our previous CK1 crystal structures (22, 23), residues 1 - 5 and 223 - 226 were disordered, and Val^{10} was modeled with disallowed backbone torsion angles in both molecules A and B. All remaining amino acid residues had appropriate backbone torsion angles, with 94% lying in the most favorable and additionally allowed regions of the Ramachandran plot (calculated for 514 non-Gly and non-Pro residues present in both molecules of the asymmetric unit).

The protein molecules were accompanied by 47 well-ordered water molecules and 11 sulfate ions (**Fig. 3**), four of which occupied the S1 and S2 sites described previously (22). In molecule A, the S1 site, a pocket ringed by Arg^{130}, Lys^{159}, Lys^{175}, Lys^{176}, and Asp^{194}, was modeled with an additional sulfate (S6), whereas in molecule B it was modeled with a single sulfate ion in association with two water molecules. The six remaining sulfates occupied three sites in each molecule. The S3/S4 sites were located within a pocket formed by the sidechains of Arg^{162}, Lys^{167}, His^{169}, and Arg^{197}, and correspond to a tungstate binding site identified previously in the mammalian Ckiδ model (1CKJ; residue 401). The remaining sulfates occupied a novel site (S5) formed at the junction of the two protein molecules by the sidechains of Arg^{243} and His^{258} and the mainchain nitrogen of Asn^{35}.

CK1 Inhibition is Specific for the E Geometric Isomer of IC261 - Each protein molecule contained clear electron density for the two aromatic rings of IC261, and for the olefinic linker connecting them, leading to unambiguous placement within the model (**Fig. 4A**). At 2.8 Å resolution, the bound

conformation of IC261 refined as the E geometric isomer with near ideal bond lengths and angles. The olefinic bond was within 1.9 ± 0.8° of planarity, whereas the dihedral angle between the two aromatic rings averaged 56.0 ± 2.1° (in both molecules of the asymmetric unit), which was within 6.0° of the angle predicted by energy minimization. This conformation was stabilized by van der Waals contact (bondlength = 3.1 Å) between the edge of the aromatic oxindole nucleus (C4) and methoxy O2'.

Electron density for IC261 was contained entirely within the nucleotide-binding cleft, formed by the junction between the alpha-helix rich C-terminal domain and the smaller, beta sheet-containing N-terminal domain. The oxindole nucleus occupied the pocket previously identified as P1 (23) which normally hosts the adenine ring of ATP (in 1CSN), and the isoquinoline nucleus of inhibitor CKI7 (in 2CSN). It was oriented so that its carbonyl group extended toward the interior of the binding cleft to within 4.5 Å of the phenol moiety of Tyr^{59} (**Fig. 4B**). When all three structures were superpositioned, the adenine and isoquinoline nuclei were nearly coplanar, whereas the oxindole nucleus differed by ≈15°. Moreover, the hydrophobic contacts made between CK1 and the oxindole nucleus were nearly identical to those described previously for the adenine nucleus (22), and the ring nitrogen of oxindole (N1) was within 1 Å of adenine N1 and isoquinoline N2. Despite these similarities, closer inspection revealed that the oxindole nucleus did not fill the P1 site as fully as did adenine, and that different modes of binding could be distinguished in the two molecules of the asymmetric unit (**Fig. 5A**). In molecule A, the electropositive edge of aromatic C6 (45) was bonded to mainchain carbonyl of Gly^{89} through a water molecule (water 412). A similar interaction was observed between N3 of ATP, water 412, and Gly^{89} in model 1CSN (22). In this position, however, N1 of the oxindole nucleus was outside hydrogen bonding distance of the mainchain nitrogen of Leu^{88}. In molecule B, no water-mediated bridge to Gly^{89} was observed, but N1 was within hydrogen bonding distance (3.4 Å) of both the mainchain nitrogen of Leu^{88} and the carbonyl of Asp^{86}. These data suggest that the oxindole nucleus binds the P1 site of CK1 through a subset of the same pharmacophores that bind and position the adenine and isoquinoline nuclei of ATP and CKI7 (**Fig. 5B**).

An olefinic bridge connects the trimethoxy phenyl moiety of IC261 to the oxindole nucleus. This moiety was located at the rear of the ATP binding pocket in van der Waals contact with Val^{153}. The trimethoxy phenyl moiety itself extended into that portion of the nucleotide-binding cleft previously identified as the P3 region, which normally binds the phosphate groups of ATP (22, 23). There, the O4' methoxy group of IC261 occupied a position nearly identical to the O2 atom of ATP alpha phosphate in model 1CSN. In contrast to ATP, however, the aromatic ring of IC261was held in position by two hydrophobic surfaces formed by the glycine-rich loop (in particular the sidechain of Ile^{26}) on one side and by Val^{153} and the aliphatic sidechain of Asp^{154} on the other. In addition, hydrogen bonds formed between the methoxy groups of the ring and CK1 (**Fig. 5A**). As mentioned above, methoxy O2' interacts with the electropositive edge of the oxindole nucleus. Methoxy O4' was within hydrogen bonding distance of both atom OD2 of catalytic residue Asp^{154} and the mainchain nitrogen of Ser^{22}, whereas methoxy O6' made contact with the NZ atom of the catalytic residue Lys^{41}.

Although contacts between IC261 and CK1 were limited, binding was accompanied by formation of an extended network of hydrogen and electrostatic bonds among CK1 residues Ser^{22}, Phe^{23}, Lys^{41}, Glu^{43}, Glu^{55}, Tyr^{59}, and Asp^{154} (**Fig. 5A**). In molecule B, this network expanded to include side chains of Asp^{131} and Asn^{136}. These data are consistent with the binding energy of IC261 being used to stabilize a network of interactions that span both N- and C-terminal domains of CK1.

IC261 is a Conformation-selective Inhibitor – Although IC261 was bound completely within the ATP-binding cleft, it proved consistently impossible to seed crystals in the trigonal space group corresponding to the "closed", ATP-bound form of Cki1Δ298. This observation, along with the novel hydrogen bonding network described above, suggested that IC261 binding was accompanied by changes in Cki1Δ298 conformation. To test this hypothesis, coordinates for molecules A and B of the Cki1Δ298:IC261 binary complex were superpositioned on the established coordinates of Cki1Δ298:MgATP (1CSN). Superposition of C-terminal domains (r.m.s. deviation of Ca positions was 0.40 and 0.48 for molecules A and B, respectively) revealed a significant movement of the N-terminal

domain (residues 6 - 89) relative to the C-terminal domain (residues 90 - 298) in the two models (**Fig. 6, *top***). Within the C-terminal domain, all differences in C_a positions (Δc_a) in excess of 1 Å were localized to loops L-BC, L-78, L-9D, L-EF, and the random coil C-terminal segment. Although changes in the latter two segments were large, they appeared unrelated to ligand binding because L-EF is disordered in all CK1 crystal structures to date and the C-terminal random coil was displaced by crystal contacts. Thus movement of the remaining loops appeared to derive from ligand binding. Superposition of N-terminal domains (r.ms. deviation of Ca positions was 0.62 and 0.74 for molecules A and B, respectively) showed that $\Delta c_a > 1$ Å were limited to loops L-12 (i.e., the glycine-rich loop), L-3A, L-A4, L-45, and the random coil N-terminal segment (**Fig. 6, *bottom***). Differences in the N-terminus and L-45 derived from crystal packing constraints, whereas movement of the other segments resulted from ligand binding. Together these data are consistent with the IC261 binding being accompanied by a rigid body rotation of the N-terminal domain relative to the C-terminal domain and by ancillary changes in six surface loops.

To quantify the rotation, the C-terminal domains of Cki1Δ298:IC261and Cki1Δ298:MgATP (1CSN) were superpositioned and the relative movement of the N-terminal domain analyzed with the program HINGEFIND (39). Although only 56% identical in amino acid sequence to *S. pombe* Cki1Δ298, mammalian CK1 (1CKJ) was included in the analysis so as to provide a model of CK1 in a non- liganded conformation. Analysis of the pair 1CSN/1CKJ revealed that MgATP substrate binding is associated with a 12° rigid-body rotation around an axis lying perpendicular to the ATP-binding pocket (**Fig. 7**). In contrast, the pair 1CSN/Cki1Δ298:IC261 were related by a rotation angle of only 7.0 ± 1.6° (mean ± range of molecules A and B) along an axis similar but not identical to that found for 1CSN/1CKJ. These data show that the IC261-bound conformation of CK1 is distinct and lies approximately midway between its non- liganded and ATP-bound conformations.

In light of this rigid body rotation, the changes in conformation of the six surface loops identified above were reassessed. On closer inspection, most changes were related to the rigid body rotation as

was found by comparing non- liganded and ATP-bound forms of CK1 (data not shown). In the C-terminal domain, L-78 moved because the sidechain of Arg^{141} hydrogen bonds with Asn^{37}, a residue that rotates as part of the N-terminal domain. L-BC appeared to move relative to the C-terminal domain because Asp^{94} no longer bound the ribose moiety of ATP through water, whereas residues 179-180 of L-9D moved as a result of Lys^{133} no longer making contact with the gamma phosphate of ATP. In the N-terminal domain, L-A4 appeared to move relative to the N-terminal domain because it stayed in contact with the C-terminal domain. In contrast, movement of both the glycine-rich loop and L-3A were related to each other but distinct from the rigid body rotation. Residues of the glycine-rich loop made direct contact with inhibitor, stabilizing the loop so that Phe^{23} bonded to the sidechain of Glu^{43} instead of Asp^{48}, as it does in the ATP-bound structure. Because L-3A makes direct contact with the glycine-rich loop, different G-loop conformations lead to different conformations of L-3A in the two structures.

These data are consistent with the E geometric isomer of IC261 stabilizing a unique conformation of CK1 so that an extended network of hydrogen and electrostatic bonds spanning the N- and C-terminal domains of the enzyme can form and render it inactive.

Discussion

IC261 is a new CK1-selective inhibitor with up to one order of magnitude greater affinity for certain CK1 isoforms than the isoquinoline sulfonamide inhibitor, CKI7. It is uncharged at physiological pH and can diffuse across cell membranes. Indeed, IC261 has been shown to inhibit Ckiδ in intact murine SV3T3 cells (46). The selectivity and affinity of IC261 for CK1 isoforms stems from an induced fit mechanism. It binds a subset of the substrate-binding pharmacophores lying in the nucleotide binding cleft resulting in stabilization of CK1 in a conformation that is midway between the unliganded and nucleotide-bound forms of the enzyme. This conformation is further stabilized by additional movement of the glycine-rich loop, which makes contact with IC261 and simultaneously participates in a novel hydrogen and electrostatic bond network involving aromatic, charged, and polar amino acid residues spanning both domains. The stability of this network of delocalized interactions decreases the dissociation rate of the inhibitor, resulting in a measurable decrease in apparent IC_{50} for members of the CK1 family relative to other protein kinases. Although Cki1Δ298 was crystallized from solutions containing both E and Z geometric isomers of IC261, the results presented here suggest that the E isomer is the energetically favored inhibitory form.

In contrast, it was shown previously that inhibition of receptor tyrosine kinases, such as FGFR, could be achieved with 3-substituted indolin-2-one derivatives in the Z conformation, such as SU4984 and SU5402 (43). Crystal structures of these ligands in complex with FGFR kinase revealed, however, that binding was accompanied by significant strain in ligand conformation. For example, both SU4984 (2.4 Å resolution) and SU5402 (2.5 Å resolution) were modeled with the olefinic bond bridging the oxindole and R group possessing substantial single bond character (the olefinic bonds in these models averaged 1.50 ± 0.05 Å in length, which is nearly 0.2 Å longer than ideal double-bond length). Moreover, these bonds were distorted from planarity an average of 26.8 ± 2.0° and 12.7 ± 2.1° in the models of SU4984 and SU5402, respectively, corresponding to an energy penalty of up to 2.5 kcal/mol. Binding of

SU5402 also led to a significant reduction in beta sheet conformation of amino acid residues of the glycine-rich loop. In contrast, the structure of Cki1Δ298:IC261complex had near ideal bond lengths and angles for IC261 without modification of protein secondary structure. Distortion of both ligand and polypeptide in FGFR kinase complexes may have resulted from soaking existing crystals of apoenzyme in ligand rather than resorting to *de novo* crystal growth of inhibitor:enzyme binary complex as was done here. Indeed, we were unable to prepare IC261:Cki1Δ298 crystals in trigonal morphology (i.e., the fully closed conformation of CK1) by microseeding, suggesting it would have been difficult to obtain the IC261-bound conformation by merely soaking existing crystals in IC261.

Structure activity relationship data proves that receptor tyrosine kinases can accommodate E as well as Z geometric isomers of 3-substituted indolin-2-one depending on the properties of substituent groups (44). Comparison of the SU4984/5402 and IC261 crystal structures reveals this can be accomplished by flipping of the indolin-2-one nucleus so that the carbonyl moiety at the 2 position points either toward the nucleotide binding pocket, as the E isomer of IC261 does in complex with CK1, or outward toward solvent, as do the Z geometric isomers of SU4984/5402 in complex with FGFR (**Fig. 8**). In either conformation, the indolin-2-one nucleus is within 15° of being coplanar with the adenine ring of ATP substrate, and retains the ability to hydrogen bond mainchain atoms in the hinge region (23) through its N1 nitrogen. We showed previously that flipping of the ring occupying the P1 site contributes to the selectivity of isoquinoline sulfonamides for individual protein kinases (23, 47).

Pharmacological modulation of protein conformation has been shown to be an effective mechanism for controlling protein activity (e.g., 48). Because all protein kinases examined to date are capable of undergoing the conformational changes described here, conformational inhibition may emerge as a general strategy for controlling protein kinase activity. Although the N- and C-terminal lobes of most protein kinases are thought to move relative to each other along a defined pathway (49), both the precise location of axes of rotation and the extent of rotation differs among protein kinases (reviewed by 50). For example, the rotation axis of CK1 lies perpendicular to the nucleotide binding site (51), whereas that

for PKA lies parallel to Helix E located in the C-terminal domain (52). In addition to differences in axis location and degree of rotation, conformations that are intermediate between liganded and non- liganded can be exploited by inhibitors as shown here for CK1 and as shown previously for PKA (53, 54).

The mechanism of inhibition found here shows promise for developing CK1-isoform selective antagonists. Although such reagents would be useful for examining the role of CK1 isoforms in cell regulation, the practicality of ATP-competitive inhibitors for therapeutics has been questioned owing to the high intracellular concentrations of inhibitor needed to overcome physiological levels of ATP (55). Therefore the non-ATP competitive CK1 antagonists (Class II) identified in our initial screens are also of great interest. Elucidating the mechanism of action of the latter reagents may yield valuable information applicable to the protein kinase family as a whole.

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FOOTNOTES

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Atomic coordinates and structure factors have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Databank under PDB # 1EH4 (http://www.rcsb.org/).

[1]The abbreviations used are: AD, Alzheimer's disease; AMP-PCP, β, γ-methyleneadenosine 5'-triphosphate; CK1, casein kinase-1; CKI7, N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide; FGFR, fibroblast growth factor receptor; IC261, 3-[(2,4,6-trimethoxyphenyl)methylidenyl]-indolin-2-one; r.m.s., root mean square; SU4984, 3-[4-(1-formylpiperazin-4-yl)benzylidenyl]-indolin-2-one; SU5402, 3-[(3-(2-carboxyethyl)-4-methylpyrrol-2-yl)methylidenyl]-indolin-2-one; PKA, cAMP-dependent protein kinase catalytic subunit

Figure Legends

Fig. 1: IC261 Structure and nomenclature. *A*, IC261 is a 3-substituted indolin-2-one derivative with anti-protein kinase inhibitory activity. E and Z geometric isomers of IC261 differ by the position of the trimethoxyphenyl moiety relative to the olefinic bond marked by an *asterisk*. The E geometric isomer is shown. *B*, When subjected to thin layer chromatography, IC261 splits into two principal species migrating with R_f values of 0.18 and 0.26. On the basis of NMR analysis, these represent the E and Z geometric isomers of IC261, with the former being the major species. *Ori*, origin. Arrow, direction of solvent migration.

Fig. 2: IC261 is a CK1-selective, ATP-competitive protein kinase inhibitor. *A*, Purified samples of protein kinases Ckiα (•), Ckiδ (□), Ckiε (×), $p34^{cdc2}$ (o), $p55^{fyn}$ (◆), and PKA (Δ) were incubated in the presence of protein substrate, 10 μM ATP, and varying concentrations of IC261 (0.09, 0.5, 2.5, 12.5, 33, 66, and 100 μM). Plots of % control activity remaining versus IC261 concentration show that IC261 inhibits the closely related Ckiδ and Ckiε isoforms selectively, with an IC_{50} (dotted line) of approximately 1 μM. This value is ≈10-fold lower than that for Ckiα, and at least 2-orders of magnitude lower than those estimated for $p34^{cdc2}$, $p55^{fyn}$, and PKA. *B*, The truncated catalytic domain of Cki1 from *S. pombe* (Cki1Δ298) was assayed in the presence of variable concentrations of ATP substrate (10, 15, 20, 40, 60, 80, and 100 μM) at constant concentration of casein substrate (2 mg/ml) and either 0 (■) or 20 μM (o) IC261. The inhibition pattern (increase in K_{mATP} with little change in V_{max}) confirms that IC261 is a competitive inhibitor of ATP substrate. In contrast IC3608, a representative Class II inhibitor analyzed at 100 μM (□), showed a noncompetitive inhibition pattern (decrease in V_{max} with little change in K_{mATP}).

Fig. 3: Overall structure of the IC261/Cki1Δ298 binary complex. The complex crystallized in space group $P6_1$, with the asymmetric unit containing two protein molecules (labeled A and B) arranged head-

to-tail. Each polypeptide chain folded into an N-terminal domain, containing 5 antiparallel β-strands (β1 – 5) and one α-helix (αA), and into a C-terminal domain containing 4 antiparallel β–strands (β6 – 9) and 8 α-helicies (αB – I) as described previously (22, 23). The final model also contained 11 sulfate ions (S1 – S6, shown in green), 47 water molecules (shown in red), and two IC261 molecules distributed between the protein molecules A and B.

Fig. 4: IC261 binds Cki1Δ298 in the nucleotide substrate cleft. IC261 electron density was displayed in stereo using TURBO-FRODO. *A*, positive $F_o - F_c$ difference density in the ATP-binding site of Cki1Δ298 contoured at 2.5 σ. The map was calculated after refinement of protein atoms but before placement of any inhibitor atoms (the IC261 model is displayed for reference only). The planar density corresponding to the oxindole and trimethoxyphenyl rings was best fit by IC261 in the E conformation. *B*, $2F_o - F_c$ map of the fully refined binary complex computed at 2.8 Å resolution and contoured at 1 σ. Carbon atoms are colored yellow, oxygen atoms red, and nitrogen atoms blue.

Fig. 5: Schematic representation of hydrogen and electrostatic interactions between Cki1Δ298 and ligands. The distances of all hydrogen bonds (dotted lines) = 3.4 Å in length are shown (for clarity, hydrophobic interactions are not illustrated). *A*, Binding of the E geometric isomer of IC261 to Cki1Δ298 stabilizes a network of hydrogen bonds among residues lining the nucleotide substrate binding pocket. In molecule A, the network extends to include hydrogen bonds between OD2 of Asp^{131} and OG of Ser^{22} and ND2 of Asn^{136}. IC261 interacts directly with the network through hydrogen bonds between its trimethoxyphenyl moiety and the sidechains of Lys^{41}, Ser^{22}, and Asp^{154}. In contrast, the oxindole nucleus binds primarily through hydrophobic interactions with the sidechains of Ile^{18}, Ile^{26}, Ala^{39}, Leu^{128}, and Val^{153}. In molecule A, $water^{412}$ bridges the carbonyl of Gly^{89} with the edge of the oxindole ring. This interaction was not observed in molecule B. Instead, N1 of the oxindole ring made hydrogen bonds to the mainchain nitrogen of Leu^{88} and the carbonyl group of Asp^{86}. *B*, in the Cki1Δ298:MgATP complex (22), binding is mediated by many of the same residues that contact IC261.

But instead of an extended network of interactions, more direct (or water-mediated) hydrogen bonds exist between ligand and protein.

Fig. 6: Conformational changes accompany Cki1Δ298 binding of IC261. Distances between equivalent a-carbon atom positions in Cki1Δ298 when complexed with MgATP versus inhibitor IC261 were calculated after *top*, superposition of C-terminal domains, and *bottom*, superposition of N-terminal domains, and plotted as a function of residue number. Boxes delineate secondary structure elements (black, β-strands 1-9; gray, α-helices A-I). The source of $\Delta C_a > 1$ Å are described in the text.

Fig. 7: IC261 binding is accompanied by a rigid body rotation of domains. C-terminal domains of CK1 bound with ATP substrate (1CSN; red), with IC261 (yellow), and without ligand (1CKJ; blue) were superpositoned as described in Experimental Procedures. The rotation axes required to align the N-terminal β-sheet of 1CKJ (blue) and IC261 binary complex (yellow) with that of 1CSN were then calculated using HINGEFIND as 12° and 7.0 ± 1.6° (mean ± range of molecules A and B), respectively. The alignment of 1CSN and CkiΔ298:IC261 was accompanied by a projection angle of 9.8 ± 1.1° (mean ± range of molecules A and B), consistent with a rigid-body rotation (39).

Fig. 8: Protein kinases bind both E and Z geometric isomers of indolin-2-one derviatives. *A*, model of the E geometric isomer of IC261 (yellow) derived from the IC261: Cki1Δ298 binary structure. The relative location of ATP (purple) after superpositioning of IC261:Cki1Δ298 binary complex and 1CSN models is shown for orientation. *B*, model of Z geometric isomer of SU4984 (yellow) in complex with FGFR kinase (1AGW). The relative location of ATP analog AMP-PCP (purple) after superpositioning of SU4984:FGFR and AMP-PCP:FGFR kinase structures (25) is shown for orientation (purple). Although the oxindole ring occupies the adenine binding pocket in both models, the rings are flipped relative to one another.

Table I

Data collection and refinement statistics

Data collection	
Unit cell dimensions (Å)	$a = b = 113.5$; $c = 110.4$
Space group	$P6_1$
Data collection temperature (°C)	-140
Reflections	
Maximum resolution (Å)	2.8
measured, unique	98304, 18625
[a]Data completeness (%)	
Overall, outer shell	95.5, 76.4
[b]R_{merge} (%)	10.9
Refinement statistics	
Atoms	
Protein, solvent, sulfate, ligand	4770, 47, 55, 46
R-factor	
[c]R_{cryst} (R_{free})	22.4 (30.5)
R.m.s.deviations from ideals	
bond lengths (Å)	0.008
bond angles (°)	1.463
B-factors (Å^2)	
Main, side, sulfate, solvent, ligand	26.6, 26.5, 42.5, 17.6, 43.6

[a]Outer shell: 2.97 – 2.80 Å.

[b]$R_{merge} = \Sigma|I_i - \langle I \rangle|/\Sigma|I_i|$, where I_i is the intensity of the ith observation and $\langle I \rangle$ is the mean intensity of the reflection, summed over all reflections.

[c]R-factor $= \Sigma||F_o| - |F_c||/\Sigma|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. R_{cryst} was calculated from the 90% of reflections used in refinement and R_{free} was calculated from the remaining 10%.

Figure 1

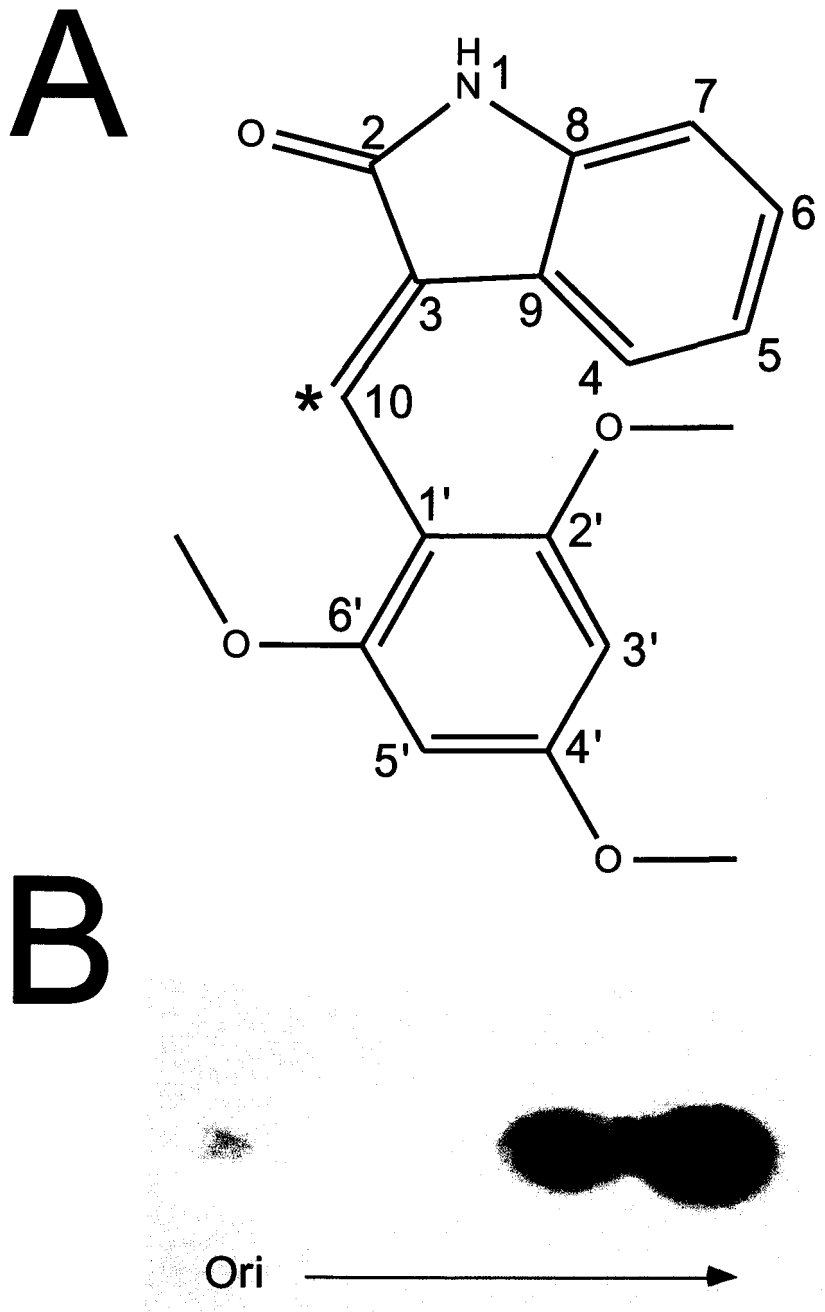

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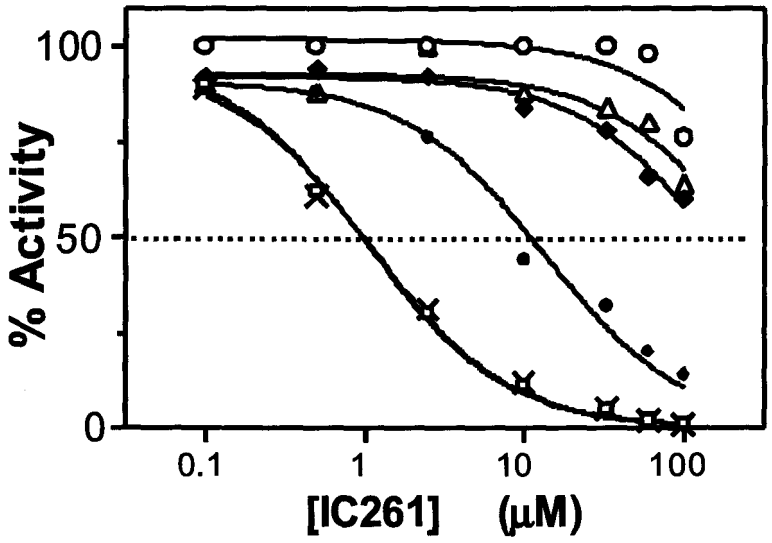
Figure 2



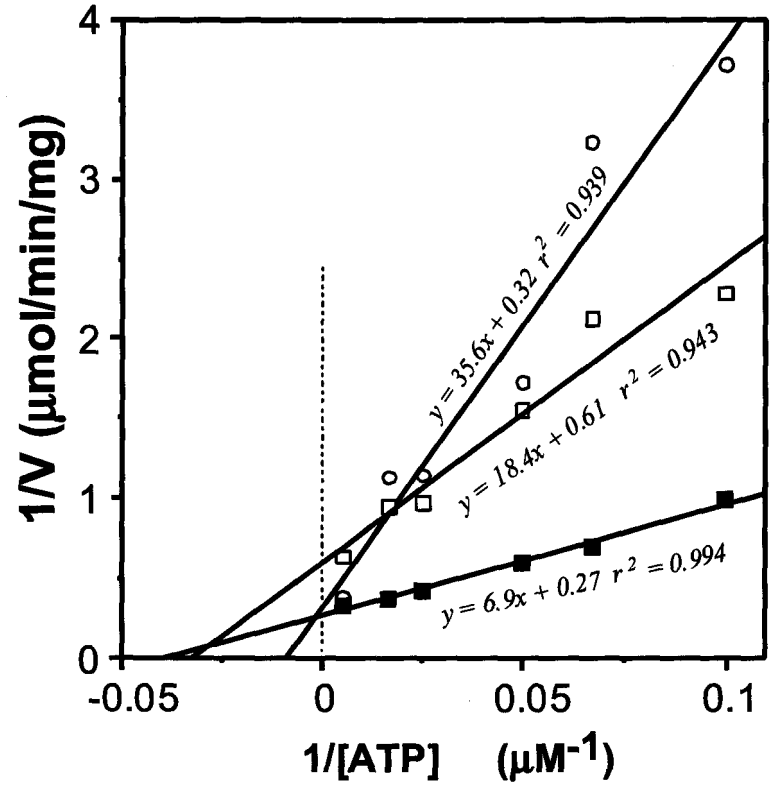


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Figure 3

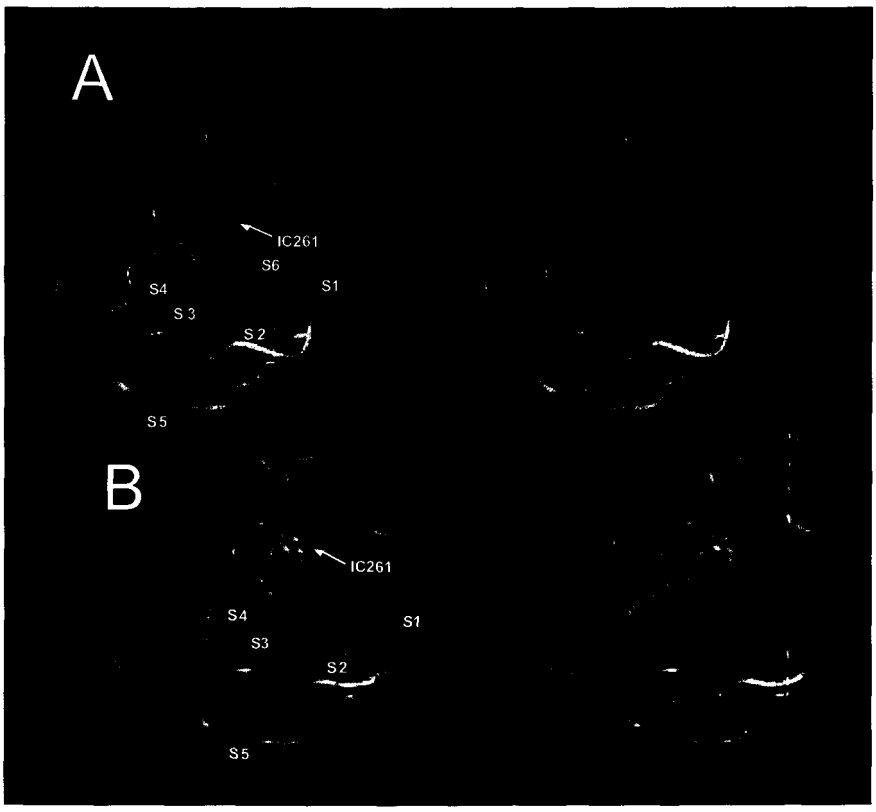

Figure 4

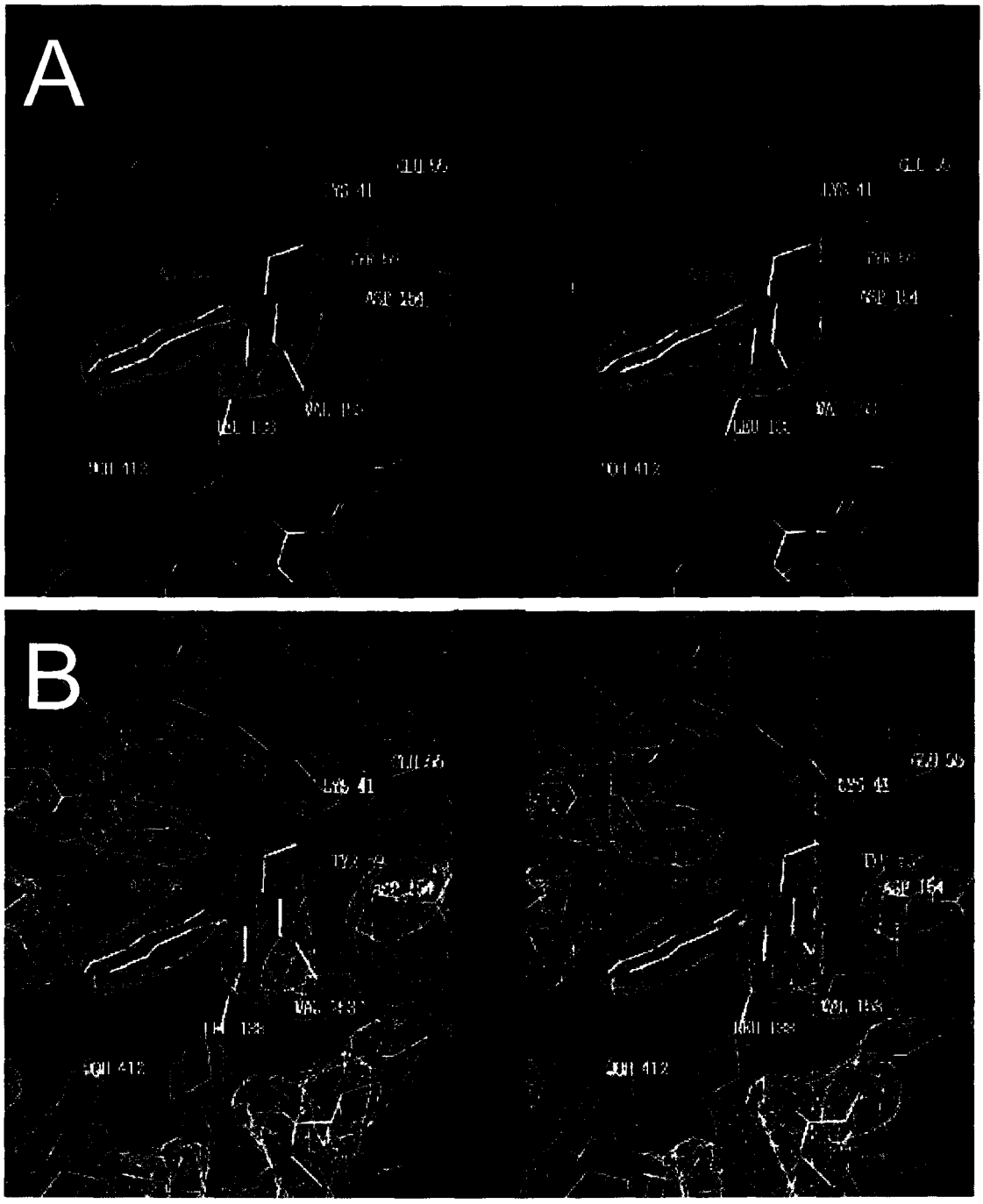

Figure 5

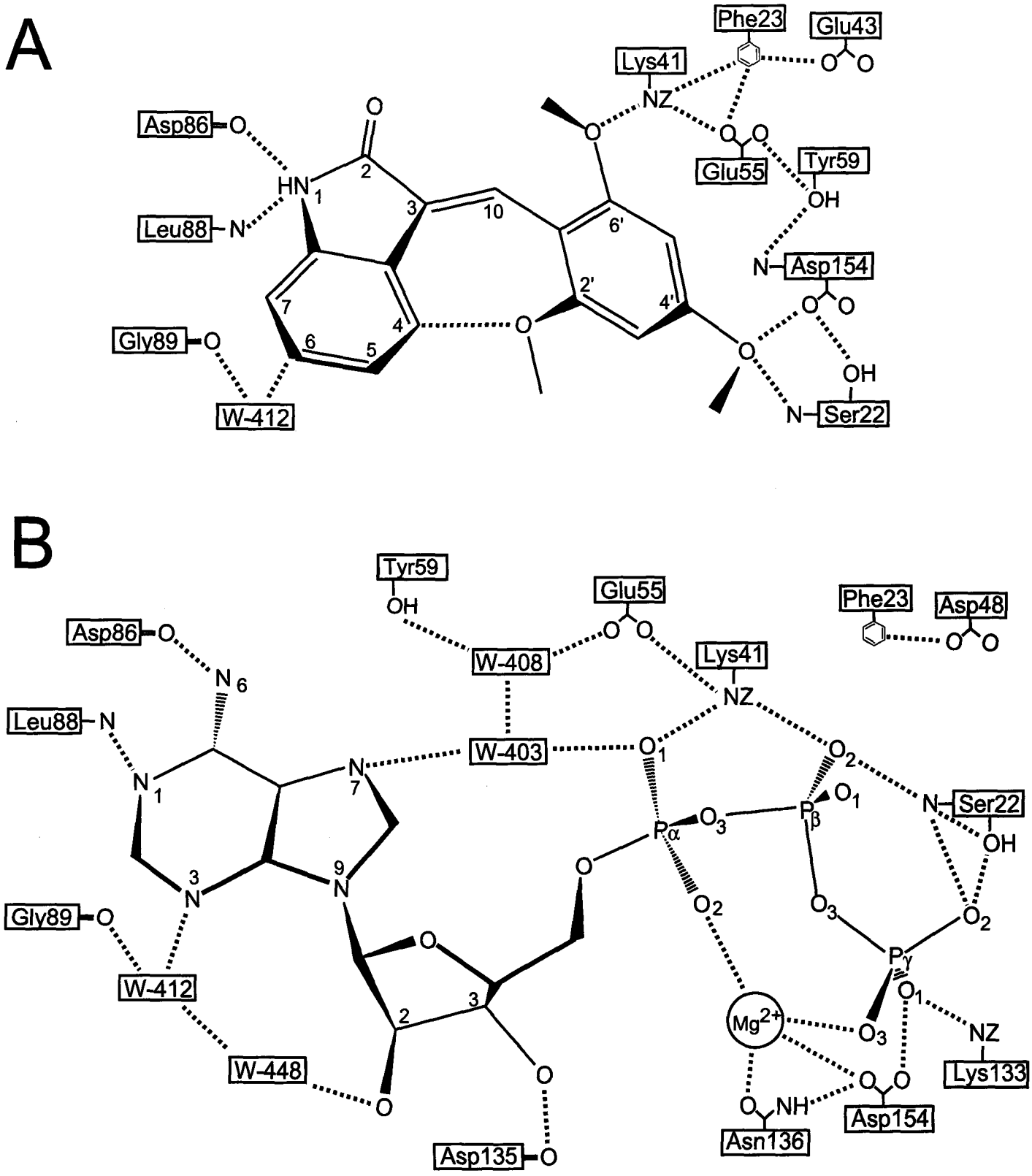

Figure 6

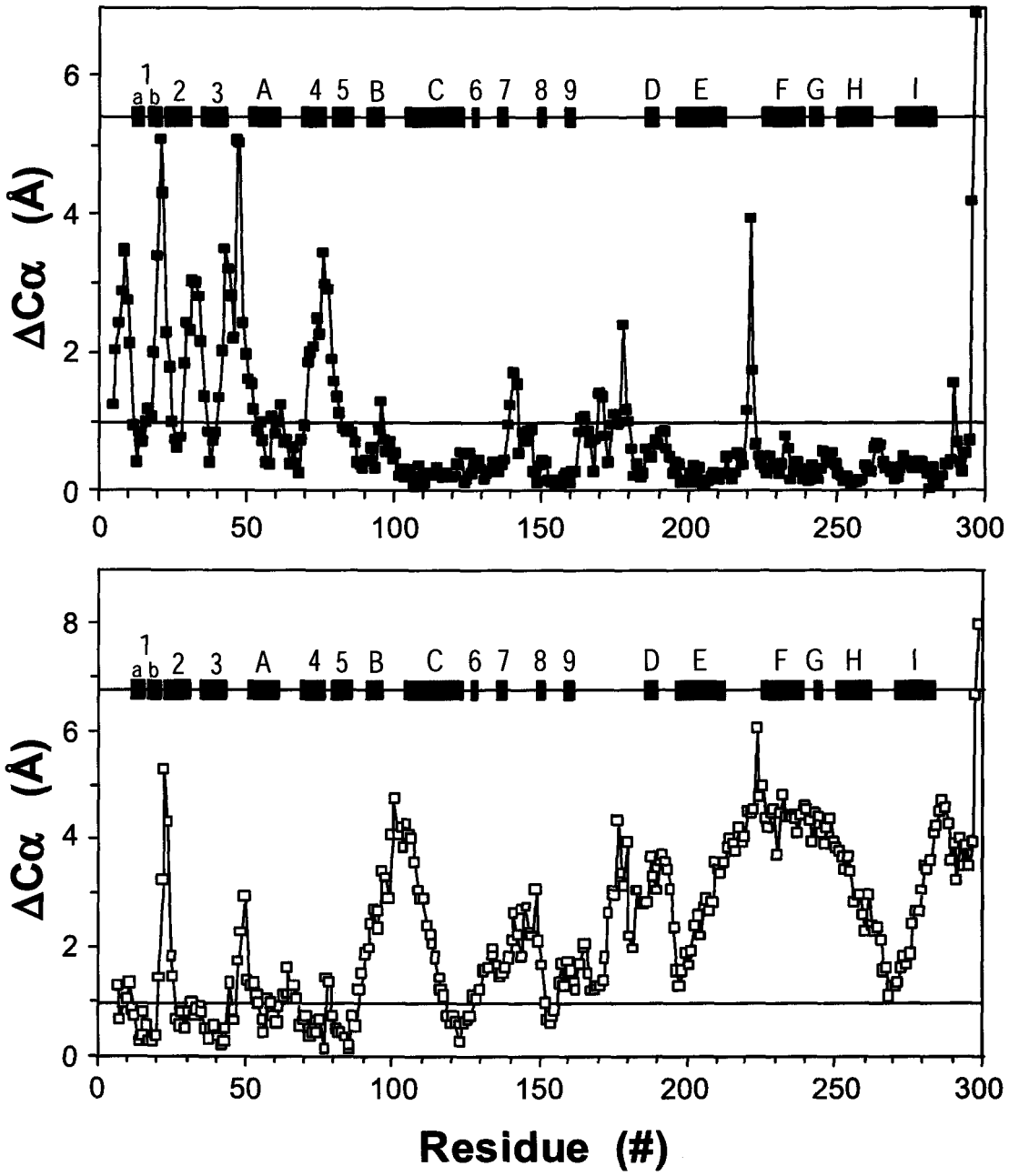

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Figure 7

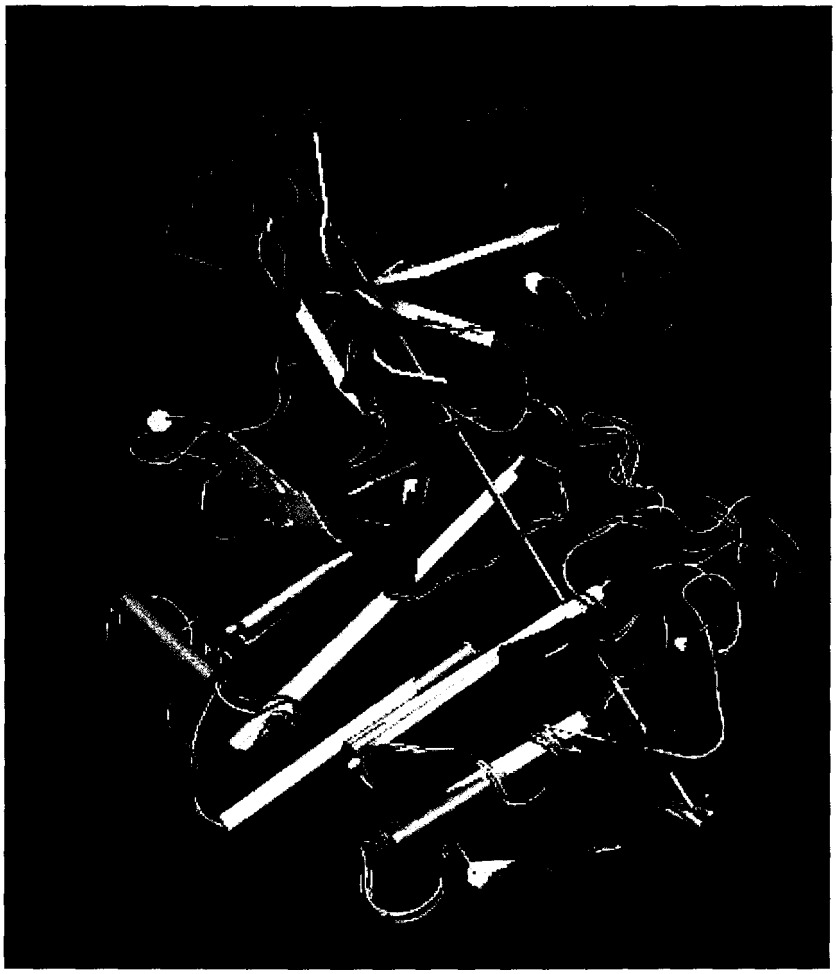

Figure 8